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LABORATORY INVESTIGATION

# Goodpasture's epitope in development of experimental autoimmune glomerulonephritis in rats

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**Goodpasture's epitope in development of experimental autoimmune glomerulonephritis in rats.** The Goodpasture's epitope (GP) has recently been localized to the last 36 AA of the non-collagenous (NC1) domain of the $\alpha_3$ chain of type IV collagen [$\alpha_3$(IV)]. Since $\alpha_3$(IV) induces glomerulonephritis (GN) in rats and rabbits, the purpose of the present study was to determine if the GP epitope itself could induce GN. We immunized rats with synthetic peptides of GP epitope, 36-mer, alone or as protein conjugates. Rats immunized with bovine GBM served as positive controls. Peptide immunized rats developed high titer antibodies to peptides, but only unconjugated 36-mer induced antibody against human and bovine GBM, but not to rat GBM. Acidic residues and the full length 36-mer were important in production of GBM reactive antibodies. Positive controls developed antibody to GBM without reactivity against 36-mer, had IgG and fibrin on the basement membrane, GN and proteinuria. Kidney eluted antibody was reactive with rat, bovine, and human GBM but not 36-mer. GN rat lymphocytes underwent blast transformation to GBM but not peptide, and peptide immunized animals responded only to the respective peptides. None of the animals immunized with GP peptide epitope, despite the development of anti-peptide antibodies or anti-GBM antibodies, developed any *in vivo* fixation of antibody to the GBM, abnormal proteinuria, or GN. The present study shows that the GP epitope is sufficient to induce an immune response to the epitope, but it is not sufficient to induce GN. This demonstrates that other factors or epitopes are important in the pathogenicity of GBM induced GN in this model. These remain to be delineated.

Glomerulonephritis (GN) in humans associated with antibodies to the glomerular basement membrane (GBM) presents as a spectrum of diseases [1]. These range from asymptomatic GBM deposits with pulmonary disease to rapidly progressive glomerulonephritis (RPGN) in the absence of pulmonary symptoms, to the extreme presentation of classic Goodpasture's syndrome with RPGN and pulmonary hemorrhage. The antigenic specificity of the pathogenic antibody to kidney or lung basement membrane does not appear to be the determinant of the clinical presentation of pulmonary hemorrhage, but rather the presence or absence of insults to the lungs, such as smoking [2–4]. The most common manifestation of anti-GBM disease is either RPGN or Goodpasture's syndrome, which usually progresses rapidly to death or end-stage renal disease requiring dialysis or transplantation [5]. Transplant patients are at risk of developing recurrent disease in the graft with consequent loss of the transplant [6, 7]. Type IV collagen in the kidney, lung and several other organs are unique since they are comprised of $\alpha_1$ and $\alpha_2$ chains, but also chains recently defined as $\alpha_3$ through $\alpha_6$ [8–12]. Type IV collagen appears to contain the epitope(s) responsible for anti-GBM disease in humans as demonstrated by reactivity of sera from patients with these diseases to the non-collagenous (NC1) domain of type IV collagen [13–15]. Most of this reactivity is directed to the $\alpha_3$(IV) chain of the NC1 domain, but not against other chains of GBM collagens [16–18].

The auto-antibodies which bind to Goodpasture's epitope have been peptide mapped to the last 36 amino acids (AA) of $\alpha_3$(IV) [16]. The Goodpasture's epitope is cryptic, being exposed upon disassociation of the hexamer of NC1 domains with acid, guanidium hydrochloride, and other disassociative agents [15, 19]. Furthermore, monoclonal antibodies directed to $\alpha_3$(IV) recognize subfractions of bovine and rat GBM [20] which produce experimental autoimmune glomerulonephritis (EAG) in rats [20]. Basement membrane antigens lacking chains found in GBM induce antibodies in rats to basement membrane structures; however, they do not result in proteinuria or GN [21]. Further, dimers of $\alpha_3$(IV) but not other NC1 domains can produce GN in rabbits [22], and rats (R. Kalluri, personal communication). Finally, there is a high degree of homology between human and rat $\alpha_3$(IV), including the last 36 AA which are almost identical [23]. These findings suggest that the epitope(s) responsible for clinical anti-GBM disease is localized to kidney type(IV) collagen basement membrane components, that $\alpha_3$(IV) collagen contains the epitope, and that the carboxy terminal 36 AA may well contain the nephritogenic epitope(s).

Since EAG in WKY rats can be induced by GBM antigen containing collagen chains but not by basement membrane lacking these chains, the present experiments test the hypothesis that the region of $\alpha_3$(IV) defined by peptide mapping to contain the Goodpasture's epitope is itself capable of inducing EAG in the rat model [21, 24]. Failure to induce EAG would suggest that an epitope other than the 36-mer associated with Goodpasture's epitope is responsible for EAG in the rat model, or that the 36-mer containing Goodpasture's epitope may be sufficient to induce marker antibodies but may require additional factor(s) for induction of GN. The findings of the present study suggest that the latter pertains, at least in the rat model.

## Methods

### *Materials*

*GBM.* Human and bovine kidneys were obtained at necropsy or from a local abattoir, and rat kidneys from animals sacrificed in

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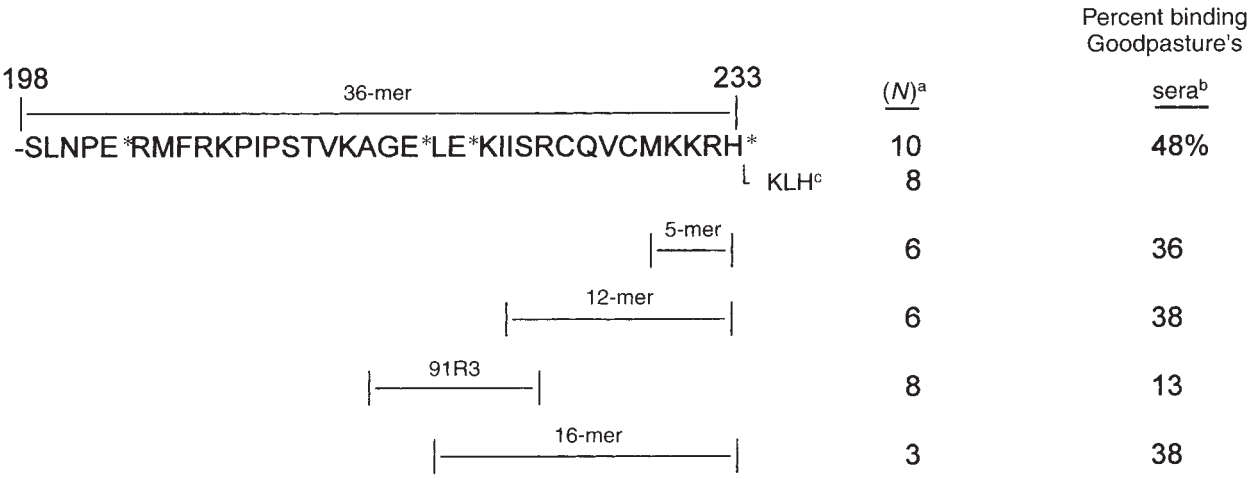


**Fig. 1.** *Amino acid sequence of peptides used to immunize rats.* The Goodpasture's epitope is contained within residues 198 to 233 of the $\alpha_3$(IV) NC1 chain. [a] The number of animals in each group is presented. [b] The approximate predicted percent binding for Goodpasture's sera by various peptides is provided in the last column (per Kalluri et al [16]). [c] KLH is the abbreviation for keyhole limpet hemocyanin. Acidic residues are indicated by an asterisk.

the Department of Comparative Medicine. Kidneys were frozen at −70°C until use and then glomeruli isolated by differential sieving as previously described [24, 25]. GBM isolated by sonication was digested with type VII collagenase (Sigma), and the resultant solubilized GBM (csGBM) was lyophilized and used as the immunizing antigen and for ELISA and immunoblotting. The term "csGBM" refers to bovine preparations unless otherwise specifically indicated.

*Hexamer.* Dissociation of the NC1 hexamer (globular domain of type IV collagen) appears to be necessary for optimal exposure of Goodpasture's epitope [15, 19]. Since our standard immunogen is csGBM containing other components of GBM, one experiment was performed with isolated NC1 hexamer to confirm that the nephritogenic antigen was related to this region of the GBM rather than other constituents of the GBM. Hexamer was isolated as described by Wieslander et al from bovine csGBM [19]. After dialysis against 2 M urea, 0.05 M Tris-HCl, pH 7.5, csGBM was chromatographed on a DE52 (Whatman) ion exchange column equilibrated with the same buffer. The globular domain, which does not bind, was dialyzed against 2 M urea, 0.05 M Tric-HCl, pH 9.0 and chromatographed on a second DE52 column in the same buffer. Bound hexamer was eluted with a 0 to 0.2 M NaCl gradient, concentrated and used to immunized rats, 100 μg/animal by our standard protocol.

### *Animals*

Male WKY rats weighing 200 grams were obtained from Harlan Sprague Dawley, maintained under standard conditions in the Vivarium and fed standard chow.

### *Synthesis of peptides*

The published sequence of the human $\alpha_3$col(IV) NC1 domain was used to synthesize the peptides used in these experiments [26]. Since our previous work strongly suggested a key role for cellular immunity in pathogenesis [24], we analyzed the AA sequence of $\alpha_3$(IV) to select short peptides which might elicit a T-cell response with minimal antibody production. Amphipathic plots were analyzed to select those most likely to be antigenic T-cell sites. Of these, peptide 91R3 (Fig. 1) fell within the terminal 36AA of the $\alpha_3$(IV) region described by Kalluri et al as containing Goodpasture's epitope [16]. We further selected the 5 mer and 12 mer peptides when 91R3 failed to produce disease. When neither the 5-mer nor 12-mer produced disease (or antibody), we synthesized the full 36-mer to use alone or after protein conjugation. The peptides were acetylated on the amino terminus and amidated on the carboxyl terminus. The last peptide designated "16-mer" comprises the carboxy terminal 16 AA with an acetylated amino terminus and free carboxyl terminus to assess the effect of a free-COOH rather than -$NH_2$ on disease induction. Peptides were synthesized using 9-fluorenylmethyloxycarbonyl chemistry on a Bioresearch 9600 automated peptide synthesizer [27]. AA were coupled to the nascent peptide chain primarily using 1-hydroxybenzotriazole active esters or if necessary symmetric anhydrides. Cysteinyl residues were protected with acetamidomethyl groups that are stable to the conditions of the cleavage and deprotection procedure [28]. The degree of coupling was quantitated with ninhydrin assay of each coupling cycle [29]. Following cleavage and deprotection of the peptide from the resin, the crude peptide product was desalted by chromatography on Sephadex G-50 and then subjected to final purification by reverse phase high pressure liquid chromatography. The purity of the product was assessed by amino acid analysis on a Beckman System Gold amino acid analysis system and mass analysis on a Finnigan LaserMat mass spectrometer.

### *Immunization procedures*

EAG develops in 100% of rats immunized by our standard protocol: 20 μg rat, bovine or human GBM suspended in 0.1 M acetic acid or after reduction, and alkylation, that is, iodoacetamide, or 1:40 β mercaptoethanol plus *Bordetella pertussis* intraperitoneally [24]. Antigen is emulsified 1:1 with CFA $H_{37}$Ra and given as a single footpad injection in 0.1 ml volume. Rats were divided into groups to receive various types of immunization by standard protocol, substituting peptide for csGBM as indicated. Group I rats were immunized with 100 μg of 36-mer. Group II was immunized with 36-mer conjugated to keyhole limpet hemocyanin (KLH) by the same regimen as described in Group I. Conjugation of peptide to carrier proteins (KLH and ovalbumin, (ova) was

accomplished using 1-ethyl-3-(3-di methlaminopropyl) carbodiimide (EDC) [30]. Peptide (1 mg/ml) was dissolved in deionized water, EDC added to a final concentration of 10 mg/ml, and the pH adjusted to 5.0. After five minutes, an equal volume of carrier protein was added to give a final molar ratio peptide to carrier protein of 40:1. Molecular weights of carrier proteins used in calculations: KLH 1,000,000; ova 45,000. Solutions were stirred for two hours at room temperature in a siliconized flask, then 1.0 M sodium acetate added to give a final pH of 4.2. The solution was stirred again for one hour, then dialyzed against PBS at 4°C overnight. Controls in this group received KLH with CFA. Group III rats received 16-mer, 50 μg/rat by standard protocol. Group IV rats were immunized with bovine csGBM. Group V rats were immunized with rat csGBM by standard protocol rather than bovine csGBM used for all other csGBM immunizations. Group VI rats were immunized by standard protocol using bovine hexamer.

Positive control animals (Group IV), as well as negative control animals, (standard protocol without antigen) were included within each peptide immunization experiment. Abnormal proteinuria and circulating anti-GBM antibodies appear by three to four weeks in GBM immunized rats with EAG.

### *Total urinary protein (TUP)*

Animals were placed in metabolic cages and 24-hour urine samples were collected weekly for TUP determinations. The latter were done using 3% sulfosalicylic acid with bovine serum albumin (BSA) as standard. [24] The upper limit of normal for 24 hour TUP in rats in our laboratory is 10 mg [31]. Animals were followed for six weeks.

### *Histopathology*

After the fifth urine collection for TUP, animals were sacrificed and sections of kidney taken for examination. For light microscopy, tissue was fixed in 10% buffered formalin and 3 μm sections were examined after staining with hematoxylin and eosin [24, 25]. Immunofluorescence studies of kidney sections were performed using commercial mono-specific fluorescein labeled anti-sera to rat IgG, C3 and fibrin (Cappel) [24, 25, 31]. The intensity of the deposits was semiquantitatively graded as 0 to 4+ [24, 25, 31]. Indirect immunofluorescence was performed on normal kidney tissue obtained in the operating room from patients having a nephrectomy and were snap frozen immediately in dry ice isopentane. Rat kidney used for indirect fluorescence was perfused with 50 ml of warm phosphate buffered saline (PBS) containing 1% BSA. Indirect assays of sera were performed at dilutions of 1:40 to 1:100 on 4 μm kidney sections as previously described [24, 25].

### *Antibody assays*

Sera from normal control subjects and from patients with Goodpasture's syndrome, and experimental animals were examined by indirect immunofluorescence and by ELISA using Immunolon II plates (Dynatech Laboratories, Inc., Alexandria, VA, USA) coated with csGBM or peptide at a concentration of 10 μg/ml. Controls included buffer alone, blanks without second antibody and omission of antigen. Positive and negative sera were assayed. For competitive ELISAs, sera were preincubated overnight at 4°C with csGBM or 36 mer as described in **Results** (Fig. 2), and ELISAs were then performed. ELISAs were all run in duplicate.

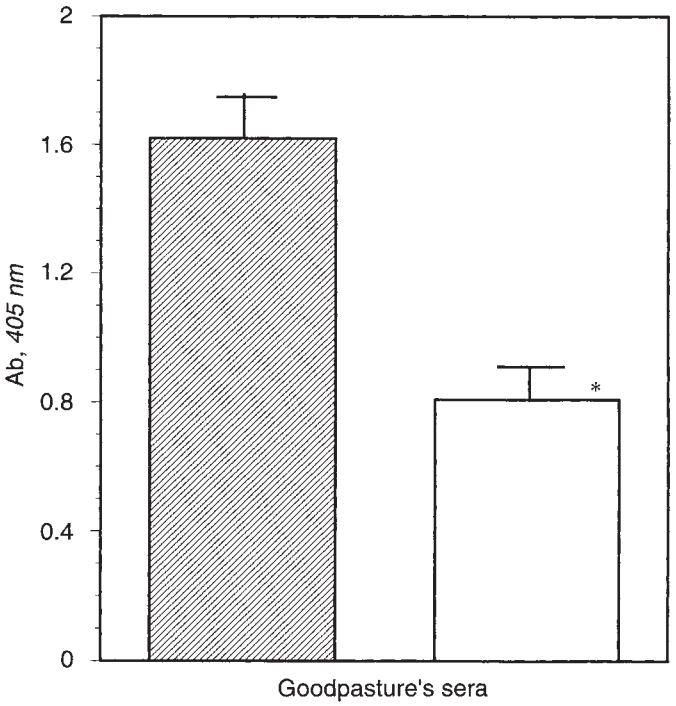


**Fig. 2.** *Binding of sera from patients with Goodpasture's syndrome to human GBM and 36-mer peptide.* Sera from nine different patients with clinical Goodpasture's syndrome were examined by ELISA with plates coated with human csGBM (▨) or 36-mer peptide (□). Corrected for normal serum background. $^*P < 0.01$.

### *Eluate*

Kidneys from nephritic and CFA control rats were eluted by the potassium iodide method and the antibodies used for ELISA and immunoblotting [32]. Briefly, 20 rat kidneys were homogenized and washed six times with PBS by centrifugation at 2000 × g at 4°C. The pellet was resuspended in 20 ml of 2.5 M potassium iodide in Tris-HCl buffer, pH 9.0 and rocked for one hour at room temperature. After centrifugation at 10,000 × g at 4°C for 20 minutes, the supernatant was collected and dialyzed extensively against PBS at 4°C. One percent BSA was added to stabilize eluted immunoglobulin.

### *Immunoblotting*

SDS-PAGE was performed using 8 to 22% gradient gels as described by Laemmli [33]. Samples included csGBM and ova-36 mer conjugate. Reduced and unreduced samples were separated electrophoretically, transferred to nitrocellulose paper, blocked with milk for two hours at room temperature and then various sera diluted 1:100 in 0.05 M PBS were overlaid for one hour. Bound IgG was demonstrated using a chemiluminescence system (Amersham) with various film exposure times to optimize banding visualization.

### *Antigen-specific lymphocyte proliferation*

Draining popliteal lymph nodes were harvested aseptically and dispersed into single cell suspensions in DMEM medium supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 1% 200 mM glutamine, 100 U penicillin, 100 μg/ml streptomycin,

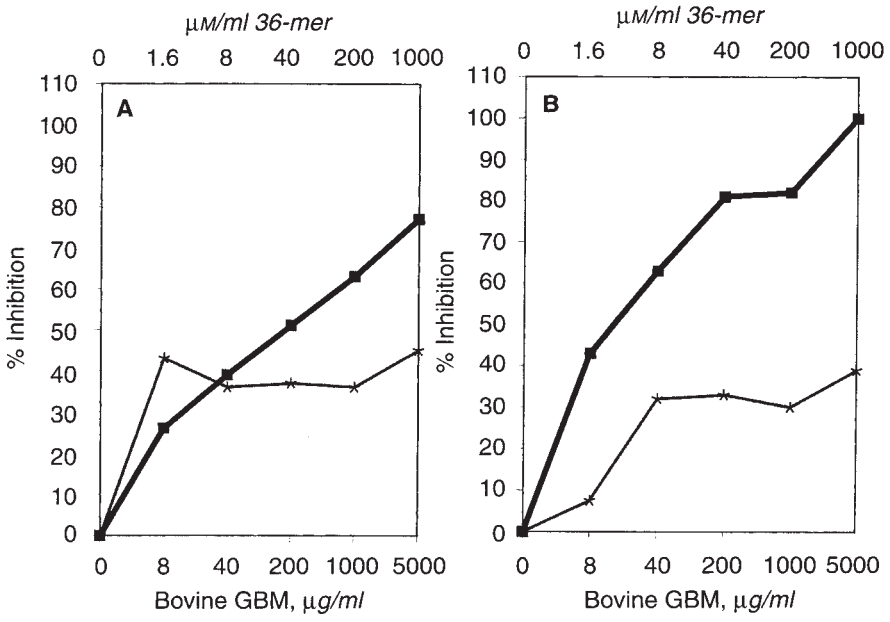


**Fig. 3.** *Competition of Goodpature's sera by 36 mer peptide and csGBM.* Goodpasture's serum competitive binding assay of human csGBM (**A**) and bovine csGBM (**B**) coated plates with increasing amounts of 36-mer (–*–) or bovine csGBM (–■–). Expressed as percent inhibition.

$5 \times 10^{-5}$ M 2-mercaptoethanol and 10% heat-inactivated fetal calf serum (Gibco, Gaithersburg, MD, USA). Cells were layered on Lymphoprep (Nygaard Company, Norway) separated, and washed three times in DMEM. Cells were enumerated by trypan blue exclusion and cultured at a concentration of $5 \times 10^4$ cells per well in a total of 200 μl in 96 well flat-bottom microtiter plates (Costar, Cambridge, MA, USA). Concanavalin A was used at a concentration of 4 μg/ml as a positive control, and medium without antigen for background. Cultures were incubated at 37°C in 5% $CO_2$ for 72 hours and then pulsed with 1 μCi of $^3$H-thymidine and cultured for an additional 16 hours. Lymphocyte proliferation assays were done in triplicate with 50 to 200 μg/ml csGBM and 10 to 100 μM concentrations of peptide. The amount of cell-associated thymidine incorporation was expressed as counts per minute (CPM) minus background counts, delta CPM (ΔCPM). A stimulation index (ratio of antigen stimulated to medium thymidine incorporation) of ≥ 2.0 was considered significant.

### *Statistical method*

Data are expressed as the mean ± SEM. All data are analyzed by the Student's *t*-test and analysis of variance [25].

## Results

### *ELISA binding of Goodpasture's auto-antibodies to csGBM and 36-mer*

Sera from nine patients with clinical pulmonary hemorrhage, RPGN, anti-GBM disease, and positive circulating antibodies by standard ELISA and indirect immunofluorescence, were assayed for antibodies against human csGBM and 36-mer (Fig. 2). All patient sera were positive against the csGBM and 36-mer in ELISA. Differences in binding are likely related to the linearity of the 36-mer sequence versus native csGBM and reduced number of intra-molecular disulfide bonds [16, 17]. Since Goodpasture's sera are known to have a spectrum of specificities to various regions of GBM as well as to different components of $\alpha_3$(IV) [18, 34, 35], studies were done to compete positive sera with 36-mer using an ELISA assay with human and bovine csGBM as substrate on plates. Anti-GBM antibody positive serum reacted strongly with both bovine and human GBM antigen, and both 36-mer and bovine GBM inhibited positive Goodpasture's sera in a dose dependent fashion (Fig. 3). The 36-mer inhibition plateaued in each assay at concentrations above 8 μM/ml (30 to 40% inhibition) while increasing quantities of bovine GBM incrementally inhibited positive Goodpasture's sera. As expected, bovine GBM competed more efficiently in ELISA's performed with bovine csGBM as the coating antigen than when human GBM was used, with essentially complete inhibition at 5,000 μg/ml of bovine csGBM. The plateau of inhibition with 36-mer with both the human and bovine csGBM substrate suggests that available antibody directed to the 36-mer epitope was completely bound at concentrations of 8 μM/ml and greater of 36-mer. Thus, synthetic 36-mer peptide bound sera from all of the positive Goodpasture's patients and competed with native antigen in ELISA.

### *Immunization with Goodpasture's epitope*

Since the last 36 AA of $\alpha_3$(IV) have been shown to contain the highest antibody binding capacity for Goodpasture's serum [16], and $\alpha_3$(IV) NC1 would appear to be the nephritogenic antigen in this rat model induced with GBM, we immunized rats to attempt to induce disease with this peptide. The results of experiments are presented in the tables and figures. Table 1 contains the ELISA of experimental sera. Table 2 describes the clinicopathology of the direct and indirect immunofluorescence, TUP excretion in 24 hours, and the presence or absence of EAG. Figure 4 is the immunoblot analysis of experimental sera versus ovalbumin$^{-36}$ mer (ova-36 mer) conjugate, and Figure 5 contains the quantitative TUP excretion.

Rats immunized with 36-mer peptide by standard protocol developed a vigorous antibody response to 36-mer and human and bovine GBM. However, there was no abnormal proteinuria, no antibody reactive to rat GBM, and no evidence of EAG.

**Table 1.** ELISA of experimental sera from animals immunized with 36-mer, 16-mer, or csGBM

| Experimental group | Immunizing antigen | (N) | Plate antigen | | | | |
|---|---|---|---|---|---|---|---|
| | | | 36-mer | 36-mer-ova | KLH | 16-mer | csGBM |
| I | 36-mer | 10 | 2.04 ± 0.10[a] | 2.10 ± 0.11 | | 2.26 ± 0.09 | 0.56 ± 0.03[a] |
| II | 36-mer-KLH | 8 | 1.99 ± 0.13[a] | 2.41 ± 0.07 | 2.20 ± 0.02 | 0.14 ± 0.01[b] | 0.06 ± 0.02[c] |
| III | 16-mer | 3 | 0.64 ± 0.25 | | | 2.22 ± 0.04 | 0.09 ± 0.00[c] |
| IV | csGBM | 11 | 0.22 ± 0.03 | | | 0.19 ± 0.02[b] | 1.92 ± 0.15 |

Abbreviation is KLH, keyhole limpet hemocyanin.
[a] $P < 0.01$ vs. csGBM, 16-mer
[b] $P < 0.01$ vs. 16-mer, 36-mer
[c] $P < 0.01$ vs. 36-mer, csGBM

**Table 2.** Clinicopathologic findings in rats immunized with peptide or csGBM

| Experimental group | (N) | Immunizing antigen | Direct IgG/fibrin[a] | Gn[b] | ↑ TUP[c] | Indirect kidney[d] Human | Rat |
|---|---|---|---|---|---|---|---|
| I | 10 | 36-mer | – | – | – | +++ | – |
| II | 8 | 36-mer-KLH | – | – | – | – | – |
| III | 3 | 16-mer | – | – | – | – | – |
| IV | 18 | csGBM | +/+ | + | + | ++++ | – |

[a] Renal tissue in immunized rates
[b] EAG, experimental autoimmune glomerulonephritis
[c] TUP, Total urinary protein
[d] Sera from immunized rats examined by indirect fluorescence on normal human and rat kidney

We reasoned that cross linking the peptide to a carrier protein might enhance its immunogenicity. Therefore, 36-mer was coupled to KLH and animals were immunized with the peptide-carrier protein or carrier protein alone. These animals developed a strong antibody response to 36-mer and the carrier protein, but not to GBM *in vitro* or *in vivo*, nor GN nor abnormal proteinuria.

### *Influence of carboxy terminus*

The previous experiments had been performed with 36-mer that was acetylated at the amino terminus and amidated at the carboxyl terminus. Since cross linking via carbamylation destroys carboxyl termini and resulted in failure to elicit an antibody response compared to peptide alone with BME, we next constructed a peptide comprising the terminal 16 AA of $\alpha_3$(IV) with an acetylated amino terminal end and a free carboxy terminus. This portion of the final 36 AA of $\alpha_3$(IV) accounts for 79% of the Goodpasture's serum binding capacity observed with Goodpasture's peptide epitopes [16]. Immunization of rats with this 16-mer resulted in the development of high titer antibodies to the peptide and to 36-mer in ELISA (free peptide) but not Western blot (conjugated peptide). There was no antibody reactivity to csGBM in ELISA, nor to human, bovine or rat kidney by indirect fluorescence. None of these animals developed antibody on the GBM, abnormal proteinuria or GN.

### *Control rats*

None of the CFA negative control rats developed antibody or disease. Positive controls, bovine csGBM immunized rats, all developed high titer circulating antibody to GBM in ELISA and Western blot which fixed to human and bovine GBM by indirect fluorescence on kidney tissue sections. These rats had IgG deposited along the GBM, fibrin deposits with proliferative GN and crescents as previously described, and progressive proteinuria (Fig. 5). Rats immunized with hexamer all developed GN indistinguishable from those immunized with csGBM. Despite the presence of antibody in kidney biopsies of the rats and the formation of circulating antibodies reactive with human and bovine GBM, circulating antibodies did not bind to normal

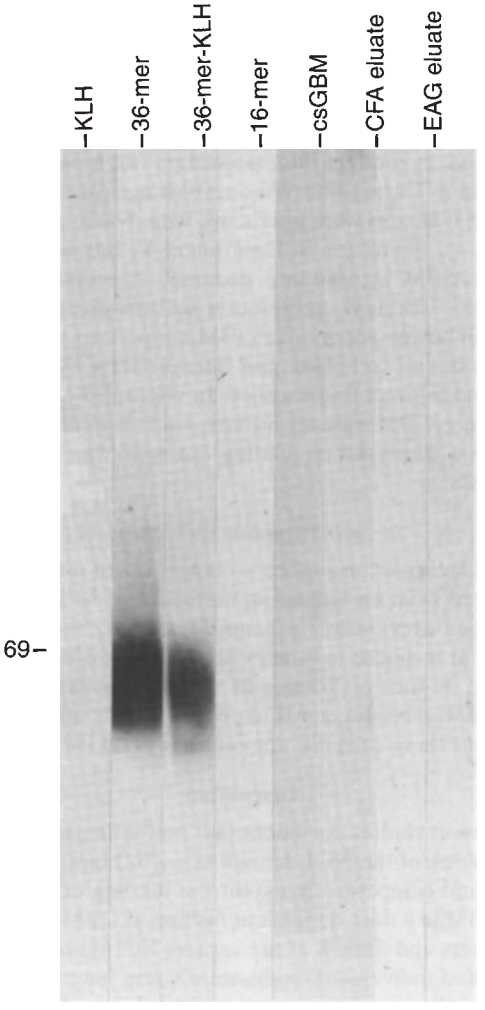


**Fig. 4.** *Immunoblot of sera from rats immunized with 36 mer or csGBM.* Immunoblot of sera from various experimental groups versus ova-36 mer conjugate, 1 μg/lane, SDS-PAGE. Size marker (kDa) is indicated. Sera used for blotting are listed above each lane. Last lane, normal rat serum.

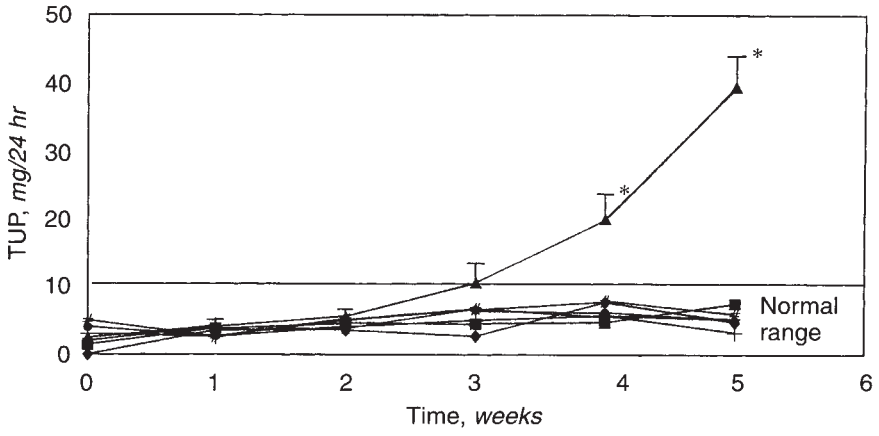


**Fig. 5.** *Development of abnormal total urinary protein (TUP) in rats immunized with bovine csGBM or peptides.* None of the rats in the experimental groups immunized with peptides developed abnormal proteinuria (> 10 mg/24 hr). Symbols are: (■) 91R3; (#) 5-mer; (●) 12-mer; (+) 16-mer; (*) 36-mer; (◇) 36-mer-KLH; (▲) csGBM. *$P < 0.01$ versus CFA controls or peptide immunized rats.

perfused rat kidney. However, eluates from nephritic kidneys were positive by indirect fluorescence on rat, human, and bovine kidney, and in ELISA and Western blot against all three species GBM. Renal eluates were unreactive with ova-36-mer in Western blots (Fig. 4). Therefore, in Experiment V, rats were immunized with rat csGBM by standard protocol. All animals developed typical EAG with heavy proteinuria indistinguishable from EAG observed in bovine and human GBM immunized rats. Circulating antibodies bound to bovine and human GBM by Western blot, ELISA, and indirect fluorescence. In contradistinction to bovine and human csGBM immunized rats, animals immunized with rat csGBM now displayed circulating antibody which bound to rat GBM *in vitro*.

### *In vitro thymidine incorporation*

Blast transformation studies were performed using lymph node cells derived from an animal immunized against bovine csGBM. As described previously, rat lymphocytes underwent blast transformation in response to human and bovine GBM (Table 3) [24], but not to 36-mer or 16-mer as the stimulating antigen. Each peptide elicited blastogenesis to cells from rats immunized to the peptide, but these cells did not respond to csGBM.

## Discussion

The present studies document that synthetic peptides spanning the last 36 AA of the NC1 domain of $\alpha_3$(IV) bind Goodpasture's antibody and competitively inhibit the binding of Goodpasture's sera to GBM in a dose dependent fashion. csGBM consists mostly of monomers and dimers of the various NC1 domains of type IV collagen, and individual Goodpasture's sera have different specificities for other components of csGBM than $\alpha_3$(IV) NC1 alone [18, 34, 35]. In addition, the tertiary structure of native csGBM cannot be reproduced by synthetic peptide. Nonetheless, the inhibition of Goodpasture's sera by 36-mer in the present studies was comparable to that reported by Kalluri et al using a competitive binding assay of peptide versus purified $\alpha_3$ NC1 domain [16]. In those studies, the percent inhibition of this region of $\alpha_3$(IV) NC1 was approximately 35 to 50%, similar to the degree of inhibition observed here. Thus our peptides appear to contain the Goodpasture's epitope as described by others [16].

Evidence cited suggests that the nephritogenic antigen for Goodpasture's syndrome and EAG in rats resides in the NC1 domain of the $\alpha_3$(IV) collagen chain. Antibody binding studies of isolated $\alpha_3$ NC1 monomers and dimers and peptide mapping imply that the terminal 36AA of NC1 $\alpha_3$(IV) may be the pathogenic antigen [22]. However, others have suggested that additional regions of $\alpha_3$(IV) may be targeted by anti-GBM autoantibodies, especially at the amino terminal portion of $\alpha_3$(IV) NC1 [36]. Peptide binding studies of Kalluri et al [22] and the competitive binding studies presented here (Fig. 3), also support the possibility that other epitopes may be involved.

Although monomers of $\alpha_3$(IV) NC1 have not been shown to be pathogenic, isolated dimers of $\alpha_3$(IV) NC1 induce EAG while basement membrane preparations lacking $\alpha_3$(IV) are non-nephritogenic [21, 22]. Since immunization of rats with human, bovine, or rat csGBM results in the production of severe EAG and the terminal 36AA of $\alpha_3$(IV) NC1 contains the Goodpasture's epitope [20, 24], we anticipated that this peptide would produce EAG. Immunization with 36-mer peptide alone or conjugated to KLH did result in high titer antibodies to 36-mer as well as any carrier protein but only animals immunized with 36-mer peptide without KLH developed antibodies cross reactive to bovine and human GBM.

Cross linking of 36-mer to KLH by the carbodiimide method, which utilizes only the carboxyl termini of acid residues [30], induced high titer antibody against 36-mer and conjugate. However, this completely blocked development of antibodies cross reactive with GBM. The last 36 AA of $\alpha_3$(IV) NC1 contains four acidic residues available for conjugation by the carbodiimide method. We routinely prepare our synthetic peptides with an amidated carboxyl terminus, so three interior acidic residues would be available for cross linking. Since carboxy terminus amidated 36-mer failed to induce EAG despite development of antibodies to GBM, and because cross linking to carrier protein by carbamylation alters the acidic residues and abolished binding to GBM, we postulated that the acid terminus might be key for a nephritogenic antibody response. Further, most antibody binding to the terminal 36 AA of $\alpha_3$(IV) NC1 is localized to the last 5 AA with a carboxy terminus [16]. We therefore repeated immunizations using the terminal 16 amino acids with a carboxyl ending. These animals developed a vigorous antibody response to peptide but failed to develop antibodies to GBM or GN. The 16-mer immunized rats did develop an antibody response to 36-mer in ELISA, but this was low titer and was unreactive against ova-36-mer conjugate in Western blot. Furthermore, 16-mer rats did not produce antibodies to human and bovine GBM as seen with

**Table 3.** Antigen specific proliferation of rat lymphocytes from animals immunized with 36-mer, 16-mer, or csGBM

| Condition | dCPM[a] | | |
|---|---|---|---|
| | 36-mer | 16-mer | csGBM |
| Medium | 4,846 ± 490 | 5,227 ± 2,023 | 6,066 ± 2,179 |
| Bovine GBM | 3,003 ± 412 (0.6)[b] | 3,698 ± 879 (0.7) | 31,519 ± 1,782 (5.2) |
| Human GBM | ND[c] | ND | 26,393 ± 1,826 (4.4) |
| 36-mer | 46,540 ± 5,133 (9.6) | 1,366 ± 352 (0.3) | 10,929 ± 1,232 (1.8) |
| 16-mer | 5,828 ± 1,713 (1.2) | 14,560 ± 2,486 (2.8) | 5,826 ± 1,990 (1.0) |

[a] dCPM, delta CPM (antigen specific CPM minus background CPM)
[b] Stimulation index, considered significant if ≥ 2.0, given in parenthesis
[c] ND, not done

36-mer rats. This implicates an important function for acidic residues in the peptide induced antibody cross reactivity to human GBM, but also suggests that the preceding 20AA are involved in this process as well. Presumably some type of conformational change occurs when all four acidic residues are cross linked by carbodiimide to KLH so resultant antibodies bind KLH and 36-mer but not human and bovine GBM. Restoration of the two acidic residues alone in the 16-mer is not sufficient to restore ability to elicit GBM cross reactive antibodies.

### *Antigen-specific lymphocyte proliferation*

Previous phenotypic studies [24] strongly suggest a role for cellular immunity in the production of this model. Animals have renal infiltrates with macrophages and lymphocytes, display delayed hypersensitivity reactions to GBM, their mononuclear cells undergo blast transformation to GBM, and development of EAG is suppressed with cyclosporine A [24, 37]. In the present studies, rats with EAG had mononuclear cell incorporation of tritiated thymidine with csGBM, as previously described, but did not respond to 36-mer or 16-mer. Rats immunized to 36-mer or 16-mer underwent blast transformation to the immunizing peptides but not csGBM. Thus, not only did the peptides fail to elicit antibody cross reactivity, but also did not produce any evidence of cross reactive cellular immunity.

None of the different peptide immunizations, regardless of the production or non-production of circulating antibody or the development of cell mediated immunity to the immunogen, resulted in the formation of antibodies *in situ* on GBM, any abnormal proteinuria or any histologic abnormalities. However, immunization of rats with native csGBM but not membrane preparations lacking $\alpha_3$(IV) NC1 results in the development of florid EAG with proteinuria [21]. NC1 hexamers also produced EAG comparable to that induced with csGBM. This may be resultant from our use of acetic acid in our immunization protocol which dissociates the hexamer [15, 19], since Kalluri et al reported that hexamer was not pathogenic in rabbits using non-dissociated hexamer [22]. Presumably some type of insult to the GBM in humans, such as hydrocarbon exposure, the effects of cell associated enzymes, etc., can expose and dissociate the hexamer, initiating the autoimmune cascade culminating in Goodpasture's syndrome. Eluates obtained from nephritic kidneys demonstrated binding to human, bovine, and rat sections and to csGBM (control eluate, 0.09 Ab units compared to nephritic eluate of 1.0 Ab units$_{405}$), but was negative in ELISA versus 36-mer (0.1 Ab units) and ova-36-mer conjugate in Western blot. Failure of EAG serum and eluate from nephritic kidneys to bind to 36-mer show that rats immunized with csGBM do not have circulating antibody to the peptide nor is antibody to the peptide bound in the kidneys on resident 36-mer or a homologous protein in the rat GBM. Yet immunization with rat csGBM produced identical disease to human and bovine csGBM, now with circulating antibodies to rat as well as human and bovine GBM. These observations demonstrate that while $\alpha_3$(IV) NC1 domain still remains a strong candidate for the nephritogenic antigen in this rat model of EAG, the synthesized 36-mer peptide containing Goodpasture's epitope, as mapped by Goodpasture's sera, is not pathogenic. Whether this is resultant from the linear nature of the synthesized peptide, from post-translational modifications that occur to the native protein *in vivo,* whether synergistic influences from other molecule(s) or effector systems are required, if the epitope required to produce EAG in rats is different from that in humans, or if epitope spreading may be necessary, all remain to be clarified.

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